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Release of 7-methylguanine residues whose imidazole rings have been opened from damaged DNA by a DNA glycosylase from Escherichia coli

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ABSTRACT

Double-stranded DNA containing 7-methylguanine residues whose imidazole rings have been opened, i.e. 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine residues, may be prepared by treatment of DNA with dimethyl sulfate followed by prolonged incubation at pH 11.4. These substituted formamidopyrimidine residues are actively removed from DNA by a DNA glycosylase present in E.coli cell extracts. The enzyme shows no apparent cofactor requirement and has a molecular weight of about 30 000. The release of ring-opened 7-methylguanine residues is due to a previously unrecognized activity, different from the three known E.coli DNA glycosylases that release uracil, 3-methyladenine, and hypoxanthine from DNA. This enzyme may serve to repair a major secondary alkylation product in DNA. In addition, it may remove nonmethylated purines, whose imidazole rings have been opened, from X-irradiated DNA.

INTRODUCTION

Several different types of nonconventional bases in DNA are recognized by DNA glycosylases that initiate a DNA repair process by catalyzing the hydrolysis of the base-sugar bond in a nucleotide residue with an altered base. In this way, uracil, 3-methyladenine and 3-ethyladenine, and hypoxanthine are released as free bases from DNA by three different enzymes (1). Since each DNA glycosylase appears highly specific for a single type of lesion, it seems likely that additional members of this class of enzymes exist which recognize other damaged bases in DNA, e.g. certain common radiation-induced products, or the various base lesions caused by spontaneous hydrolytic decay of DNA. For this reason, we have been interested in the fate of DNA purine residues whose imidazole rings have been opened.

Cleavage of the C^8-N^9 bonds of both guanine and adenine residues occurs as a consequence of exposure to ionizing radiation in the absence of oxygen (2-4). Opening of the imidazole ring leads to the conversion of a purine residue to a substituted diaminopyrimidine, which remains bound to

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deoxyribose in DNA and may act as a premutagenic lesion. Moreover, cleavage of the C^8-N^9 bond of adenine (but not guanine) occurs during alkaline hydrolysis of DNA (5,6) and a similar event could conceivably also take place at a much lower but biologically significant rate at neutral pH. Since this type of damage in DNA can occur by a number of routes, it seemed possible that living cells might be able to repair DNA containing rare guanine and adenine residues with opened imidazole rings, and that DNA glycosylases exist to deal with such lesions.

Several different types of DNA base damage occur after radiolysis or alkaline hydrolysis. Therefore, instead of employing DNA exposed to ionizing radiation or strong alkali to search for such DNA glycosylases, a model substrate containing the lesion of interest as the predominant altered base was sought. Fortunately, the major product after treatment of DNA with simple alkylating agents, 7-methylguanine, has a very alkali-labile imidazole ring which is rapidly cleaved in mild alkaline solution (7-9). The free, ring-opened base, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (here abbreviated faPy), may be released from DNA by formic acid hydrolysis or mild acidic hydrolysis (7). This chain of events is shown in Fig.1 for a 7-methyldeoxyguanosine residue in alkylated DNA. It seemed clear that it would be possible to alkylate DNA with radioactive dimethyl sulfate, followed by incubation at high pH, and thus obtain double-stranded DNA containing most of its radioactively labeled residues in the form of faPy. Here we show that such DNA is readily prepared and that it is a substrate for a previously unrecognized DNA glycosylase from E.coli.

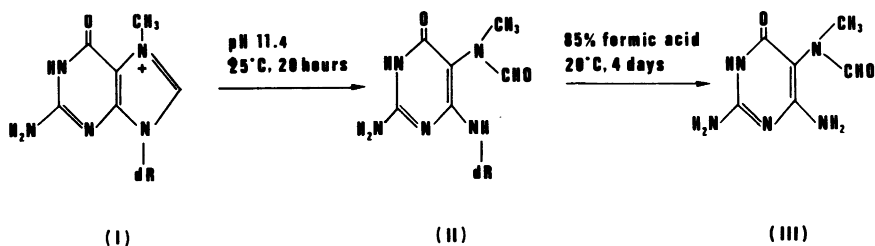

Fig. 1. Conversion of a 7-methyldeoxyguanosine residue (I) in alkylated DNA to a form with an opened imidazole ring (II) by alkali treatment, followed by release of the free altered base, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (III), by acid hydrolysis.

MATERIALS AND METHODS

Preparation of 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine. A 7-methylguanine derivative with an opened imidazole ring was made essentially according to Haines *et al.* (7). Twenty-six mg 7-methylguanosine, purchased from Sigma, were dissolved in 2 ml 2 M NH_3 and incubated at 20° for 2 hours to obtain complete imidazole ring cleavage. This ring-opened compound was then lyophilized, dissolved in 2 ml 85 % formic acid, and incubated at 20° for 4 days to achieve glycosyl bond cleavage. This material was lyophilized, redissolved in water, applied as a band to Whatman 3MM paper, and the product purified by descending paper chromatography in isopropanol/conc.NH_3/H_2O (7:1:2) for 21 hours. The ultraviolet-absorbing band was cut out, extracted with 8 ml H_2O, lyophilized, and redissolved in 1 ml H_2O. This substituted pyrimidine had the expected ultraviolet absorption spectrum and chromatographic properties of 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (7). The C^8 residue of ring-opened purines may be lost under acidic conditions (7), but no significant deformylation of the compound had occurred here as determined by analysis of its elementary composition by mass spectrometry.

Preparation of alkylated DNA containing ring-opened 7-methylguanine residues. Calf thymus DNA was purchased from Worthington and dissolved (8 mg/ml) in 0.2 M potassium cacodylate, pH 7.2, containing 1 mM EDTA. Four ml of this solution were incubated with 5 mCi of [methyl-^{3}H]dimethyl sulfate (New England Nuclear Corp., 3.95 mCi/μmole) for 1 hour at 37°. The alkylated DNA was precipitated with two volumes cold ethanol, washed with 80 % ethanol, dissolved in 4 ml of 1 M NaCl/0.01 M phosphate/1 mM EDTA, pH 7.4, and dialyzed in the cold against 0.05 M Na_2HPO_4-NaOH, pH 11.4, for 24 hours. The DNA solution was then incubated at 25°, either for 8 hours to convert about 50 % of the 7-methylguanine residues to a ring-opened form or for 20 hours to cause about 85 % imidazole ring cleavage of 7-methylguanine residues and simultaneous partial loss of 3-methyladenine residues by nonenzymatic hydrolysis. Finally, the DNA was dialyzed against 0.02 M Tris•HCl, pH 7.5/1 mM EDTA overnight and stored frozen at -20°. Such DNA preparations had specific radioactivities of 500-600 cpm/μg.

Paper chromatography. All experiments were performed by descending chromatography on Whatman 3MM paper. System I (ref.10) contained isobutyric acid /H_2O/0.1 M EDTA/conc. NH_3/toluene (160:22:3:2:20, v/v), system II was isopropanol/conc. NH_3/H_2O(7:1:2), and system III was the upper phase of ethyl acetate/n-propanol/H_2O (4:1:2). After drying of the papers, references were

localized by their ultraviolet absorption. Strips containing individual samples were then cut transversely in 1-cm pieces. Each piece was cut into small fragments, which were transferred to a scintillation counting vial. After elution with 2 ml H_2O at room temperature for 2-16 hours, 15 ml of Triton X-based scintillation fluid were added and the radioactivity of the fraction determined.

Reference compounds were faPy, 7-methylguanine (Sigma), and 3-methyladenine (Fluka AG).

Enzyme assay. The standard reaction mixture (50 µl) contains 0.1 M KCl/0.07 M Hepes•KOH, pH 7.8/1 mM EDTA/1 mM dithiothreitol/5 % glycerol/ /5 µg alkylated DNA with ring-opened 7-methylguanine residues (3000 cpm)/ /enzyme (1-10 nanounits). After 20 minutes at 37°, the reaction mixtures were chilled to 0°, and 10 µl of a 0.2 % solution of heat-denatured calf thymus DNA, 5 µl of 2 M NaCl, and 150 µl of cold ethanol were added. After 10 minutes at 0^oC, the samples were centrifuged for 20 minutes at top speed in an Eppendorf 5412 centrifuge, and 150 µl of each supernatant were recovered. In preliminary assays of column fractions, the radioactivity of this material was determined directly. In most cases, however, nonradioactive faPy was added as carrier, the volume of the solutions was reduced to about 30 µl under vacuum, and the samples were analyzed by paper chromatography in System I. One unit of enzyme catalyzes the release of 1 µmol free faPy per minute under the standard reaction conditions.

Enzyme purification. Frozen E.coli B cells were purchased from the Microbiological Research Establishment, Porton, England. Twenty grams cell paste were disrupted at -20° in a modified Hughes press and extracted with 100 ml of 0.05 M Tris•HCl, pH 8.0/1 mM EDTA/1 mM 2-mercaptoethanol. After removal of debris by centrifugation, an equal volume of 2 % streptomycin sulfate in the extraction buffer was added, and the resulting precipitate removed by centrifugation. Solid ammonium sulfate was added to 45 % saturation (1.75 M), and the precipitate was removed by centrifugation. Additional ammonium sulfate was then added to the supernatant to a final concentration of 70 % saturation (2.73 M), and the resulting precipitate was collected by centrifugation and suspended in 5 ml of 0.5 M KCl/0.05 M Tris•HCl, pH 7.5/1 mM EDTA/1 mM 2-mercaptoethanol/5 % glycerol. After dialysis overnight against this buffer, the sample was applied to a column of Sephadex G-75 (3 x 100 cm) equilibrated with the same buffer. The activity on ring-opened 7-methylguanine residues in DNA eluted as a symmetrical peak after most of the protein but before the peak of uracil-DNA glycosylase activity. The most active frac-

tions (35 ml) were pooled and dialysed overnight against a 90 % saturated ammonium sulfate solution containing 0.05 M Tris•HCl, pH 8.0/2 mM EDTA/5 mM 2-mercaptoethanol/5 % glycerol. The precipitated protein was collected by centrifugation, suspended in 2.8 ml of the gel chromatography buffer, and dialysed for 24 hours against a 50 % glycerol solution containing 0.5 M NaCl/ /0.05 M Tris•HCl, pH 7.5/1 mM EDTA/1 mM 2-mercaptoethanol. This solution, which contained 35 mg protein and 4 microunits of enzyme activity in 1.6 ml, was about 12-fold purified with respect to the DNA glycosylase activity studied here, in 50 % yield. It could be stored unfrozen at -20° for several months with little or no decrease in activity.

Other DNA glycosylases. E.coli uracil-DNA glycosylase was purified and assayed as described (11), employing phage PBS1 DNA containing radioactive uracil residues as substrate. E.coli 3-methyladenine-DNA glycosylase was purified and assayed as described (12), using DNA treated with radioactive dimethyl sulfate as substrate. Hypoxanthine-DNA glycosylase was assayed as described (13), employing poly([^{3}H]dI)•poly(dC) as substrate.

Bacterial strains. E.coli BD10 (ung^{-}), deficient in uracil-DNA glycosylase (14), was obtained from H.R.Warner. E.coli BK2012 and PK432 (tag^{-}, xth^{-}) are two strains deficient in both 3-methyladenine-DNA glycosylase and exonuclease III (1; P.Karran, T.Lindahl, I.Öfsteng, and E.Seeberg, ms. in preparation). Bacteria were grown in liquid broth and harvested in the logarithmic growth phase.

RESULTS

Properties of DNA containing faPy residues. DNA was alkylated with [^{3}H]dimethyl sulfate, hydrolyzed in 0.1 M HCl, and the radioactive products were analyzed by paper chromatography. As expected (8), a major peak of 7-methylguanine and a minor peak of 3-methyladenine were observed (Fig.2). Since two of the three previously known DNA glycosylases show a strong preference for double-stranded DNA, conditions were sought that would allow ring-opening of 7-methylguanine residues in DNA without causing alkaline denaturation of the secondary structure. Incubation at pH 11.4 and 25° for several hours was found to meet these requirements. After 8 hours of incubation, 50 % of the 7-methylguanine had been converted to faPy (Fig.2), and after 20 hours incubation 85 % conversion had occurred. Simultaneously, some 3-methyladenine was released from the DNA by hydrolysis of the very labile glycosyl bond of 3-methyl-dAMP residues and removed during the subsequent dialysis of the alkali-treated DNA (Fig.2). The slow conversion of 7-methylguanine in double-stranded

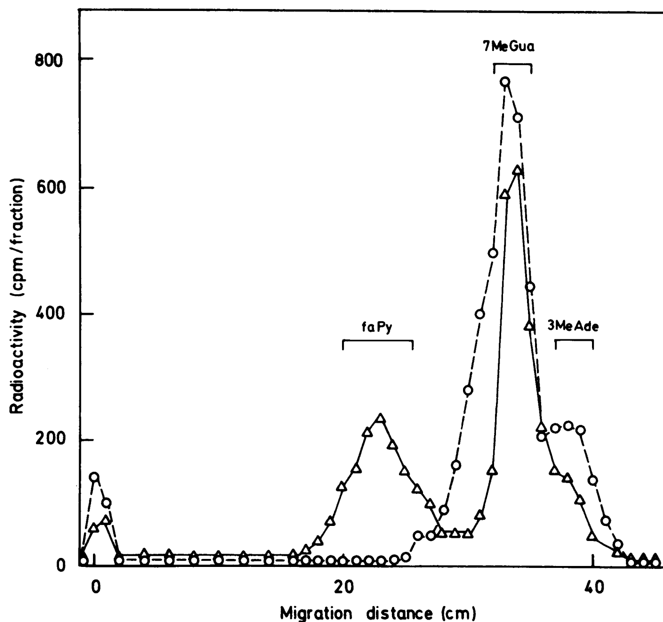

Fig. 2. Base analysis by paper chromatography of DNA alkylated with $[^3H]$dimethyl sulfate, before (O---O) and after (Δ——Δ) incubation of the DNA at pH 11.4 for 8 hours at 25° to cause partial conversion of the 7-methylguanine residues to a species with an opened imidazole ring. The DNA was hydrolysed in 0.1 M HCl for 16 hours at 37° and then chromatographed in System I for 11 hours together with authentic markers.

DNA to a compound with the chromatographic properties of faPy was confirmed in three solvent systems. System I (materials and methods) was used in most subsequent experiments because it separates faPy well from both 3-methyladenine and 7-methylguanine as well as from mononucleotides, although faPy has a tendency to give an elongated spot (Fig.2).

Enzymatic release of free faPy from DNA. Crude cell extracts of *E.coli* were found to release faPy and 3-methyladenine, but not 7-methylguanine, from alkali-treated alkylated DNA. The faPy-releasing activity was purified 12-fold by ammonium sulfate fractionation and Sephadex G-75 gel chromatography but still contained 3-methyladenine-releasing activity after these steps(Fig.3). The latter activity is due to the *E.coli* 3-methyladenine-DNA glycosylase (12). A 2800-fold purified preparation of that enzyme was found to release 3-methyladenine but not faPy from the present DNA substrate; neither 3-methyladenine nor faPy were released in significant amounts during the

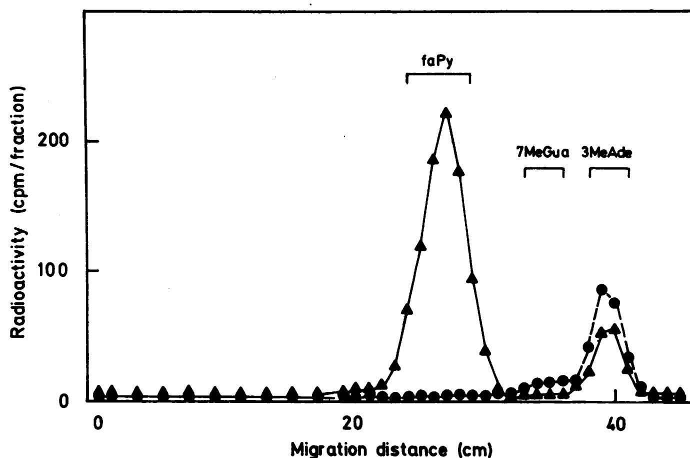

Fig. 3. Paper chromatographic analysis of ethanol soluble material released from DNA alkylated with [^{3}H]dimethyl sulfate by a partly purified E.coli DNA glycosylase fraction (17 μg protein added to the standard reaction mixture). The DNA was used as a substrate either directly after alkylation (●- -●) or after alkylation followed by pH 11.4 treatment (▲——▲). The conditions of chromatography were described in Fig.2.

standard assay in the absence of an added E.coli enzyme fraction (Fig.4). These data show that the E.coli extract contained at least two different DNA glycosylase activities, the previously characterized 3-methyladenine-DNA glycosylase and a novel activity that releases faPy from DNA. The identity of the enzymatically released, radioactive material as authentic faPy was

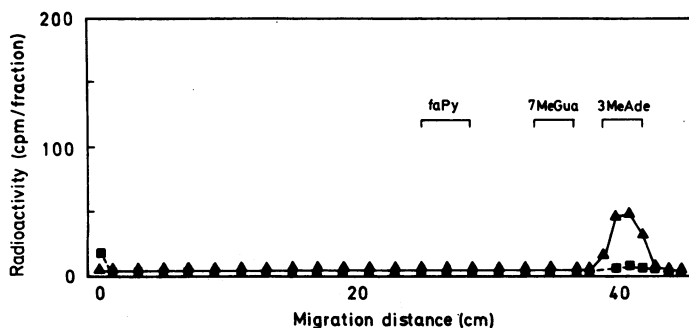

Fig. 4. Analysis of the ethanol soluble material released from alkylated and pH 11.4-treated DNA under the standard assay conditions by a large amount (0.5 microunit) of purified 3-methyladenine-DNA glycosylase (▲——▲) or by nonenzymatic hydrolysis (■- -■). Conditions as in Fig.3.

verified by paper chromatography in three different solvent systems. The enzyme fraction used here could release more than 80 % of the faPy (and the 3-methyladenine) present in the DNA substrate (Figs. 3 and 5).

General properties of the enzyme. The E.coli activity that releases faPy from alkylated, alkali-treated DNA had the properties of a (globular) protein of molecular weight about 30 000, as estimated by gel chromatography on a precalibrated Sephadex G-75 column (15). The activity had a broad pH optimum between 7.2 and 8.0. There was no detectable cofactor requirement, and the release of faPy from DNA occurred equally effectively in the presence of 1 mM EDTA (no added Mg^{2+}) or 5 mM $MgCl_2$. In a standard reaction mixture without added KCl, the release of faPy from DNA was twofold slower than in the presence of 0.1 M KCl, while increasing the KCl concentration from 0.1 M to 0.4 M also gave a similar decrease in activity. Thus, the activity is relatively insensitive to inhibition by neutral salts. The enzyme showed a strong, possibly absolute, preference for double-stranded DNA as a substrate. Alkylat-

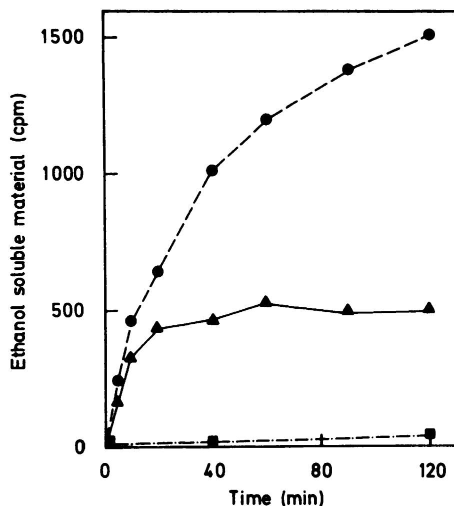

Fig. 5. Amount of ethanol soluble material released from alkylated and pH 11.4-treated DNA (50 % of the 7-methylguanine residues in ring-opened form) by the 12-fold purified DNA glycosylase fraction. Seventeen µg protein were added per 50 µl reaction mixture. The reaction was allowed to proceed for different times under the standard incubation conditions and terminated by ethanol precipitation. The reaction mixture contained either DNA (4000 cpm) and enzyme (●- -●), a smaller amount of the same DNA (1000 cpm) and enzyme (▲——▲), or DNA (4000 cpm) but no enzyme (■- -■). Chromatographic analysis of the enzymatically released material is shown in Fig.3.

ed DNA that had been incubated at pH 12.3, instead of at pH 11.4, in order to obtain both imidazole ring-opening of 7-methylguanine residues and denaturation of the double helical structure was a very poor substrate, and faPy was released at only 2 % the rate observed with the pH 11.4-treated DNA. However, the enzyme efficiently recognized and released faPy from double-stranded DNA containing small amounts of this base (Fig.5), and an apparent K_m of $2 \cdot 10^{-9}$ M for faPy residues in DNA was determined by varying the substrate concentration in the standard reaction mixture.

Activity is due to a novel DNA glycosylase. The experiments described show that E.coli cell extracts contain a DNA glycosylase activity that releases faPy in free form from DNA containing this damaged base. The activity might have been due to a previously undetected DNA glycosylase, or alternatively to the ability of a known DNA glycosylase to recognize and also release faPy residues. The following results indicate that the former alternative is the correct one.

The preparation of faPy-releasing activity used here is still relatively crude and contained 3-methyladenine-DNA glycosylase activity, raising the possibility that a new function of that enzyme was being observed. However, it was shown that a highly purified preparation of 3-methyladenine-DNA glycosylase did not release faPy from DNA (Fig.4). Further, extracts from two E.coli K-12 strains deficient in 3-methyladenine-DNA glycosylase activity, BK2012 and PK432 (tag), were found to contain normal, wild-type levels of faPy-releasing activity.

Similar observations were made with respect to uracil-DNA glycosylase. An essentially homogeneous preparation of that enzyme failed to release detectable amounts of faPy from the DNA substrate employed here (<2 % faPy released under the present standard assay conditions by 50 microunits of uracil-DNA glycosylase). A cell extract from the E.coli strain BD 10, deficient in uracil-DNA glycosylase activity (ung), had a normal, wild-type level of faPy-releasing activity. Moreover, uracil-DNA glycosylase (M=24 500) appears to be a smaller enzyme than the faPy-releasing glycosylase, and these two proteins were separated by gel chromatography on Sephadex G-75 as two distinct but partly overlapping peaks of activity.

Another E.coli DNA glycosylase, the hypoxanthine-DNA glycosylase, is not presently available in highly purified form, and mutants deficient in this enzyme have not been isolated. However, in contrast to the other known DNA glycosylases this enzyme is precipitated by relatively low concentrations of ammonium sulfate, and it could be separated from the faPy-

releasing activity studied here in this fashion. Thus, while a crude E.coli cell extract contained both hypoxanthine- and faPy-releasing activity, no hypoxanthine-DNA glycosylase activity could be detected in the 12-fold purified faPy-DNA glycosylase preparation employed for most of the present experiments. We therefore conclude that the enzymatic release of faPy from DNA does not seem to be due to any of the previously known DNA glycosylases.

DISCUSSION

Four different DNA glycosylases have now been found in E.coli. The DNA glycosylase described here, which releases a purine residue whose imidazole ring has been opened from DNA, shows similar properties to the previously known enzymes of this class in that it has a molecular weight of about 30 000 and no apparent requirement for Mg^{2+}, P_i, or other cofactors. It would appear that the various DNA glycosylases are relatively small enzymes that act by similar mechanisms, although each enzyme has a narrow substrate specificity and only recognizes a single type of altered base in DNA. In this respect, the enzyme investigated here has been shown to release effectively a 7-methylguanine residue whose imidazole ring has been opened from DNA, while it does not liberate intact 7-methylguanine residues. It is unclear at present if such a faPy residue in DNA is the "true" substrate for the enzyme. It seems reasonable, however, to assume that faPy might be recognized by a DNA glycosylase, because E.coli cells can actively repair several types of alkylation damage and remove alkylation products present in smaller quantities than 7-methylguanine, i.e. 3-methyladenine (12) and O^6-methylguanine, from their DNA, although removal of the latter lesion apparently involves an in situ alteration process rather than release of O^6-methylguanine by a DNA glycosylase (12,16). Moreover, whereas intact 7-methylguanine residues in DNA appear to be fairly innocuous lesions that are not actively released *in vivo* (17), the ring-opened form of this base would presumably be a miscoding or noncoding residue that may be generated at a significant rate by nonenzymatic hydrolysis of 7-methylguanine even at neutral pH. As an alternative, the enzyme investigated here may have as primary function the removal of ring-opened unmethylated guanine residues from DNA *in vivo*. If this were the case, the enzyme would be expected to function in X-ray repair.

An E.coli DNA glycosylase, which acts on alkali-treated DNA and releases adenine residues which have opened imidazole rings, has been observed in preliminary experiments. It is not known, however, if that activity and the enzyme activity which releases faPy as described here are contained

within the same protein. It has been noted previously that base residues with cleaved rings may be DNA lesions that are as important in radiobiology as pyrimidine dimers are in photobiology (18). However, little is presently known about the mechanisms of repair of this type of DNA damage. Since a DNA glycosylase which releases a purine derivative with an opened imidazole ring has now been found, it seems plausible that other types of fragmented base residues, e.g. the derivatives obtained by cleavage of pyrimidine rings, may also be recognized and removed by specific DNA glycosylases.

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Abbreviations used: faPy, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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